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# Short-term responses of soybean roots to individual and combinatorial effects of elevated [$CO_2$] and water deficit

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ABSTRACT

Climate change increasingly threatens plant growth and productivity. Soybean (*Glycine max*) is one of the most important crops in the world. Although its responses to increased atmospheric carbon dioxide concentration ([$CO_2$]) have been previously studied, root molecular responses to elevated [$CO_2$] (E[$CO_2$]) or the combination/interaction of E[$CO_2$] and water deficit remain unexamined. In this study, we evaluated the individual and combinatory effects of E[$CO_2$] and water deficit on the physiology and root molecular responses in soybean. Plants growing under E[$CO_2$] exhibited increased photosynthesis that resulted in a higher biomass, plant height, and leaf area. E[$CO_2$] decreased the transcripts levels of genes involved in iron uptake and transport, antioxidant activity, secondary metabolism and defense, and stress responses in roots. When plants grown under E[$CO_2$] are treated with instantaneous water deficit, E[$CO_2$] reverted the expression of water deficit-induced genes related to stress, defense, transport and nutrient deficiency. Furthermore, the interaction of both treatments uniquely affected the expression of genes. Both physiological and transcriptomic analyses demonstrated that E[$CO_2$] may mitigate the negative effects of water deficit on the soybean roots. In addition, the identification of genes that are modulated by the interaction of E[$CO_2$] and water deficit suggests an emergent response that is triggered only under this specific condition.

## 1. Introduction

Global climate change is driven by an increasing atmospheric carbon dioxide ($CO_2$) concentration. This leads to temperature extremes and alters precipitation patterns, impacting plant growth and productivity [1,2]. Concurrently, due to the steady global population growth, it is imperative that we increase current food production by the year 2040 by 70% [3]. Soybean (*Glycine max*) is the most economically important legume worldwide. It is widely used for human and animal consumption due to the high protein and oil contents in its seeds and serves as a source for biodiesel production [4]. The global soybean production in 2014/2015 was approximately 319 million tons, and Brazil and United States are currently the top two producers [5]. This scenario highlights the importance of understanding the physiological, biochemical, and genetic responses of soybean to elevated atmospheric concentration $CO_2$ (E[$CO_2$]) and water deficit to provide strategies to improve its adaptation to climate variables.

Atmospheric $CO_2$-equivalent concentrations have been predicted to reach 475–1313 parts per million (ppm) towards the end of the 21 st century [6]. The primary effects of E[$CO_2$] on growth have been well documented and include higher photosynthetic rates, lower stomatal conductance, lower leaf transpiration and increased night respiration [7–12]. Nevertheless, these studies have focused largely on aboveground processes. To understand other processes, such as the critical feedbacks and adjustments that occur within a plant and between plants and the soil, it is necessary to consider the effects on roots [13]. E[$CO_2$]

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has been demonstrated to impact root physiology, including changes in root biomass, length, number, thickness, branching, longevity, mortality, and water and nutrient uptake (reviewed by Rogers [14], and Madhu and Hatfield [13]). All of these responses are influenced by growth conditions and are highly dependent on their genetic background [13,14]. However, information about the molecular responses of roots to E[$CO_2$] is largely unknown.

In addition to rising atmospheric $CO_2$, plants are susceptible to other climate factors such as heat and reduced water availability. Water deficit may reduce soybean yield by approximately 40%, affecting all stages of plant growth and development as well as seed quality [15,16]. The root is the first organ to sense water deficit-related signals [17], which triggers rapid changes in gene expression [18]. For instance, early stages of water deficit, simulated by using a hydroponic system, induced the expression of 3089 genes in soybean roots [18]. The hydroponic growth system allows the application of acute moisture stress or instantaneous water deficit treatment what can reveal, associated with RNA-seq technology, early gene expression responses.

Previous studies with different species including barley, maize and sorghum [19–24], have demonstrated that E[$CO_2$] can alleviate the negative effects of water deficit. This alleviation occurs because under E[$CO_2$] stomatal conductance decreases, allowing plants to use water more efficiently. As consequence, net photosynthetic rates can be maintained for longer periods, leading to a better biomass accumulation compared to plants at A[$CO_2$] under water stress [19–24]. Furthermore, Sicher and Barnaby [23] showed that by maintaining a normal water status, E[$CO_2$] might delay the signaling processes responsible for inducing the expression of stress-related genes. In addition, E[$CO_2$] significantly attenuated the negative impact of combined heat and water deficit in *Arabidopsis thaliana* through altered genes related to redox metabolism and photorespiration [25]. However, none of these studies have focused on the combinatorial effects of E[$CO_2$] and water deficit in roots, the first plant organ to experience and respond to the stress.

In the present study, soybean roots were used as a model to investigate the effects of E[$CO_2$], singly and in combination with water deficit, on the overall gene expression. We therefore exposed soybean to a realistic climate event, i.e., water deficit under ambient and E[$CO_2$], and analyzed plant responses at the level of growth, photosynthesis and genome-wide transcriptional changes. As result of this combinatorial approach, we illuminated mechanisms underlying the plant responses to climate change, and the main effect of elevated [$CO_2$] on water deficit stress. It was found that E[$CO_2$] decreased gene expression related to transport, secondary metabolism, antioxidant activity and transcription factors. When plants grown under E[$CO_2$] are treated with instantaneous water deficit, E[$CO_2$] reverted the expression of water deficit-induced genes mainly related to stress, defense, transport and nutrient deficiency and induced the expression of genes that are not activated by the two treatments alone.

## 2. Results

### 2.1. Leaf gas exchange, growth and biomass

The net photosynthesis rate (*A*) of soybean leaves doubled in plants grown under elevated [$CO_2$] (E[$CO_2$]) compared to those grown under ambient [$CO_2$] (A[$CO_2$]) (Fig. 1A, T0). When water deficit was applied, photosynthesis at E[$CO_2$] remained similar to the initial rates, while it decreased 36.4% at A[$CO_2$] after 50 min of water deficit (Fig. 1A). Stomatal conductance ($g_s$) was 15% lower at E[$CO_2$] than under A[$CO_2$] at T0 and decreased by approximately 46% and 84% upon applying water deficit for 50 min to the roots of plants growing in E[$CO_2$] and A[$CO_2$], respectively (Fig. 1B). Water deficit imposed to the roots promoted a reduction in the fresh weight of leaves and roots (Fig. 1C, 1E), where the roots were most affected by water deficit. Fresh weight of stems was not affected by water deficit (Fig. 1D). The leaf relative water content (RWC), which directly reflects the water status of plants, displayed a significant reduction in both $CO_2$ treatments after 50 min of water deficit (Fig. 1F). Plants under A[$CO_2$] had significantly lower RWC values than plants under E[$CO_2$] at all time points. Furthermore, the differences between $CO_2$ treatments increased with time under stress (4.6% at T0, 5.8% at T25, and 6.9% at T50). The total dry biomass was 50% greater under E[$CO_2$] (data not shown). This positive response resulted from an increase of 49.3%, 61.6%, and 37.9% in the biomass of leaves, stems, and roots, respectively (Fig. 1G). E[$CO_2$] increased plant height by approximately 18% (Fig. 1H) and leaf area by approximately 43% (Fig. 1I). Conversely, E[$CO_2$] treatment did not significantly affect root length (Fig. 1J).

### 2.2. Analyses of RNA sequencing (RNA-seq) data

To explore the molecular responses in the roots of soybean plants grown under A[$CO_2$] and E[$CO_2$], samples from zero (T0) and 50 (T50) minutes of water deficit were evaluated. These time points were chosen based on the differential effects they had on physiological parameters.

Using 100 base pairs (bp) paired-end sequencing, we obtained 428,820,564 sequence reads. Each biological replicate was represented by at least 47 million reads (Table 1). The Pearson correlation between the biological replicates was high (0.98 to 0.99; Table S1). The alignment on the *G. max* v1.1 genome showed that 61.7–65.3% of the total number of reads were confined to exons, and 217,995,029 had perfect matches (OMM) in the reference genome (Table 1). An average of 19% of reads mapped to ribosomal RNA (rRNAs) and small nuclear (snRNAs), introns, splice junctions, and intergenic regions (data not shown). The total number of expressed genes was over 43,409 per sample (Table 1). Out of 56,044 protein-coding loci and 88,647 transcripts that have been predicted for the soybean genome [26], 54,175 genes were identified in this study. A total number of 40,252 expressed genes (74.5%) were common to all biological samples (data not shown).

### 2.3. Transcriptional responses of roots to E[$CO_2$]

Genome-wide expression profiling identified 302 genes that were differentially expressed between the roots of soybean plants (V3/V4 stage) grown under A[$CO_2$] and E[$CO_2$] (T0). Among them, 28 genes (9.3%) were up-regulated and 274 (90.7%) down-regulated (Table 2, Table S2). In total, 280 (92.7%) were identified as *Arabidopsis thaliana* orthologues (Table S2).

To determine which genes and pathways were relevant to soybean roots responses to E[$CO_2$], a gene set enrichment analysis (GSEA) was performed on the 302 differentially expressed. Ninety-nine biological processes were significantly enriched in the $CO_2$-regulated gene sets, and most of them were down-regulated (Table S3). The transport category was among the biological processes with the highest number of genes differentially expressed, mainly down regulated, between A[$CO_2$] and E[$CO_2$]. Genes belonging to this category encode for distinct protein transporters types such as metal ion nutrients, amino acids, oligopeptides, sucrose, lipids, general substrates, auxin, calcium, and nitrate (Fig. 2A, Table S2). In addition, processes associated with oxidation-reduction, signaling, response to biotic and abiotic stimuli, monocarboxylic acid biosynthetic metabolism, defense response, stress (water deprivation and oxidative stress), secondary metabolism (phenylpropanoid and flavonoid biosynthetic process), and nutrient level (response to nitrogen and iron) were also overrepresented (Fig. 2A, Table S3). In terms of molecular function, the overrepresented categories were related to oxidoreductase activity, transmembrane transporter activity, heme-binding, iron ion binding, ATPase activity, oxygen binding, antioxidant activity, glutathione transferase activity, and nicotianamine synthase activity (Fig. 2B, Table S3). The 302 differentially expressed soybean genes were also queried against the KOBAS database (KEGG Orthology Based Annotation System). The results of this analysis indicated that E[$CO_2$] significantly influences the expression patterns of

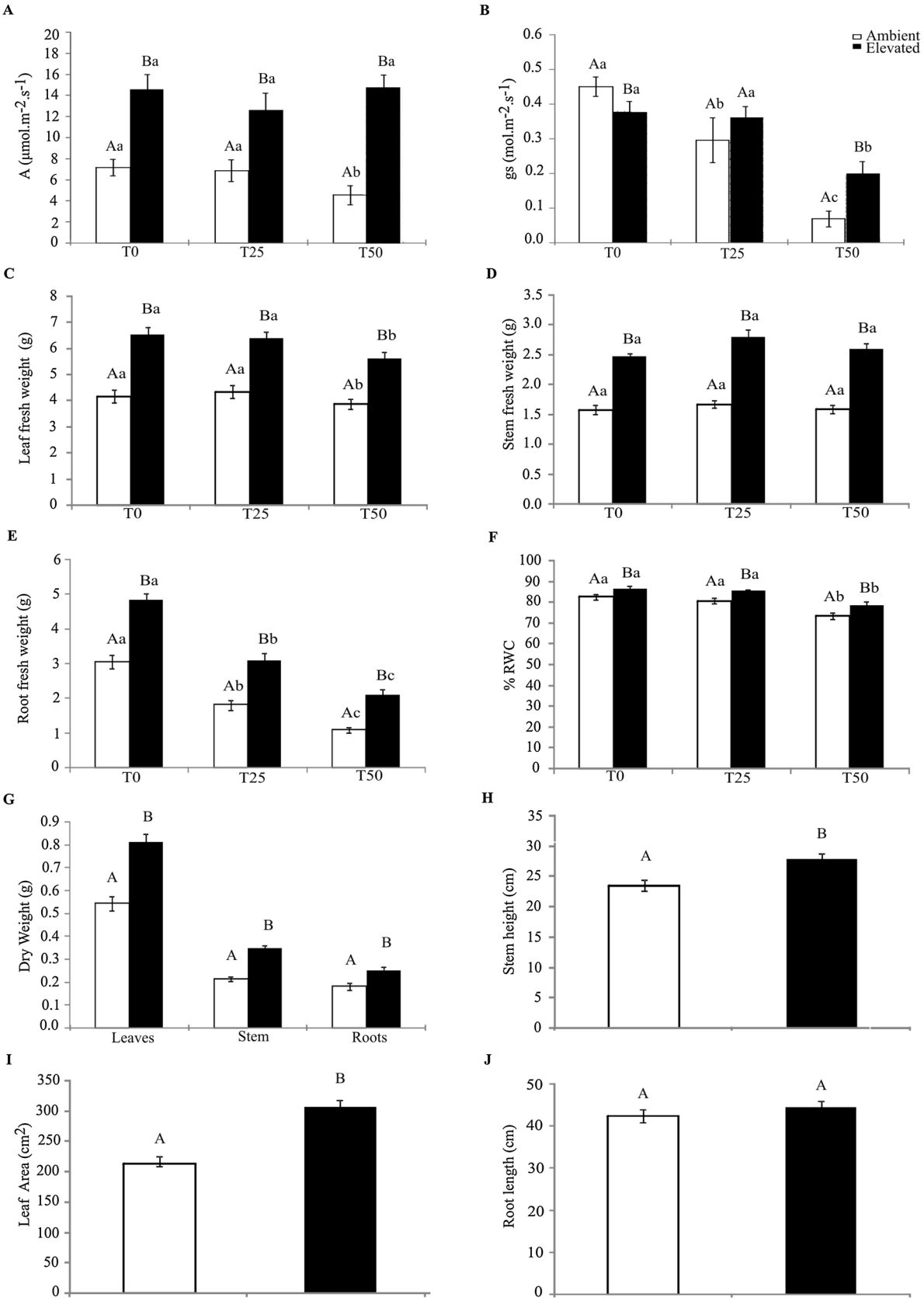


**Fig. 1.** Physiological analyses of soybean cultivated under A[$CO_2$] and E[$CO_2$] submitted to different water deficit times. **(A)** Photosynthetic rate (*A*) and **(B)** stomatal conductance ($g_s$) measured after 0, 25, and 50 min of water deficit in soybean plants cultivated in OTCs under A[$CO_2$] and E[$CO_2$]. Fresh weight of **(C)** leaves; **(D)** stem; **(E)** roots. **(F)** Relative Water Content (RWC); **(G)** dry weight; **(H)** stem height; **(I)** leaf area; (J) root lengths. The variables sampled along time (0, 25, and 50 min) were analyzed by two-way ANOVA and Tukey's honest significance difference (HSD) test. The biomass, height, and leaf area were analyzed using a one-way ANOVA and Tukey's HSD. The capital letters compare the treatments at the same time point. The lowercase compare the same treatment at different times points ($p < 0.05$).

**Table 1**
Summary of sequencing data and their mapping onto the soybean genome (*Glycine max* v1.1).

| | Total Reads[1] | Mapped Reads[2] | % of total | OMM[3] | Expressed Genes | Yield_Gbases |
|---|---|---|---|---|---|---|
| **A [CO2] -T0_1** | | | | | 43,923 | 5.3 |
| **Exon** | 53,083,219 | 33,240,588 | **62.6** | 26,891,787 | | |
| Not mapped | 53,097,534 | 9,973,968 | 18.78 | | | |
| **A [CO2] -T0_2** | | | | | 43,825 | 5.24 |
| **Exon** | 52,480,126 | 32,797,672 | **62.48** | 26,206,037 | | |
| Not mapped | 52,495,840 | 9,576,896 | 18.24 | | | |
| **E [CO2] -T0_1** | | | | | 43,430 | 5.26 |
| **Exon** | 52,624,833 | 33,165,386 | **63.01** | 26,767,655 | | |
| Not mapped | 52,636,256 | 9,316,793 | 17.70 | | | |
| **E [CO2] -T0_2** | | | | | 43,409 | 4.78 |
| **Exon** | 47,729,584 | 30,253,078 | **63.37** | 24,116,169 | | |
| Not mapped | 47,741,906 | 8,178,292 | 17.13 | | | |
| **A [CO2] -T50_1** | | | | | 44,511 | 5.58 |
| **Exon** | 55,719,168 | 35,581,272 | **63.84** | 28,841,025 | | |
| Not mapped | 55,730,908 | 9,830,225 | 17.64 | | | |
| **A [CO2] -T50_2** | | | | | 44,325 | 6.14 |
| **Exon** | 61,361,795 | 37,877,874 | **61.72** | 30,096,820 | | |
| Not mapped | 61,374,162 | 11,734,885 | 19.12 | | | |
| **E [CO2] -T50_1** | | | | | 44,174 | 5.52 |
| **Exon** | 55,094,085 | 35,428,149 | **64.28** | 28,656,906 | | |
| Not mapped | 55,112,054 | 9,622,811 | 17.46 | | | |
| **E [CO2] -T50_2** | | | | | 43,793 | 5.08 |
| **Exon** | 50,727,754 | 33,151,128 | **65.33** | 26,418,630 | | |
| Not mapped | 50,742,486 | 8,829,535 | 17.40 | | | |
| **TOTAL** | 428,820,564 | 271,495,147 | | 217,995,029 | | |

[1] Total number of reads mapped on the *G. max* genome.
[2] Percentage of reads mapped on the *G. max* genome.
[3] OMM = Number of perfect matches to the reference sequence.

**Table 2**
Number of genes differentially expressed in soybean roots under E[$CO_2$], water deficit, and interaction $CO_2$:water deficit.

| | E[$CO_2$] | Water deficit | E[$CO_2$]:water deficit |
|---|---|---|---|
| **Up-regulated** | 28 | 5193/ 3811* | 14 |
| **Down-regulated** | 274 | 8362/ 3412* | 2 |
| **Total** | 302 | 13,555/ 7223* | 16 |
| * LogFC≥ 2 | | | |

several transcripts related to secondary metabolite pathways, predominantly by down-regulating them, such as phenylpropanoid and flavonoid biosynthesis, stilbenoid, diarylheptanoid and gingerol biosynthesis, limonene and pinene degradation, betanidin degradation, phenylalanine metabolism, glutathione metabolism, and baicalein degradation (hydrogen peroxide detoxification) (Table S4). The secondary metabolism category includes *PHENYLALANINE AMMONIA-LYASE 1* (*PAL1*), *PEROXIDASE 52* (*PRX52*), and *PEROXIDASES 72* (AT5G66390). These genes mediate phenylalanine metabolism and, together with *CINNAMYL ALCOHOL DEHYDROGENASE 6 (CAD6*), participate in phenylpropanoid biosynthesis. *TRANSPARENT TESTA 4* (*TT4*) and *CYTOCHROME P450* genes (*CYP78A5*, *CYP81D3*, *CYP82C4*, and *CYP71B34)* have the expression regulated by E[$CO_2$] (Table S4). TT4 is involved in flavonoid biosynthesis that depends on phenylpropanoid synthesis. The biosynthesis of stilbenoid, diaryl heptanoid, and gingerol is represented by *CYP78A5*, *CYP81D3*, *CYP82C4*, and *CYP71B34* genes, which also participate in limonene and pinene degradation (Tables S4, S5, Fig. S1). Additionally, we observed genes that participate in iron uptake and transport, with high score associations in *FERRIC REDUCTION OXIDASE 2* (*FRO2*), *NICOTIANAMINE SYNTHASE* (*NAS1*, *NAS2*, *NAS3*), *FERRETIN 1* (*FER1*), *ZRT/ITR-like 2* (*ZIP2*), and *ZINC TRANSPORTER PRECURSOR 10* (*ZIP10*) (Fig. S1, Table S5). Genes encoding putative peroxidases (*PEROXIDASE 54* -AT5G06730, AT1 G14550) and *FERRETIN 1* (*FER1*) also participate in antioxidant activity [27; 28] as well as genes from the glutathione pathway (glutathione S-transferases, *GSTU4*, *GSTU7*, *GSTU19,* and *GSTU8*) (Tables S4, S5, Fig. S1).

To assess whether our experimental results on roots shared a common gene set in response to E[$CO_2$] with other $CO_2$ experiments, we compared the differentially expressed RNA-Seq root transcriptome with the data available for leaves of soybean [12,29] and Arabidopsis [30,31], since there is no other available information about roots. The comparison between the genes regulated in roots and leaves revealed, as expected, only a few common genes in response to E[$CO_2$] (Fig. S2, Table S6). Among the six genes that overlap between our experiment and Leakey and collaborators [12] (Fig. S2B), two encode for proteins related to oxidation-reduction processes (glutathione S-transferase and 12-oxophytodienoate reductase 2). A recent work of Jauregui and collaborators investigated the expression in roots of Arabidopsis plants grown under 800 ppm [$CO_2$] [32]. Plants were grown in hydroponic solution and, after five weeks of exposure to the $CO_2$ treatments, roots were collected and the gene expression was accessed by microarrays [32]. They identified 174 differentially expressed genes in roots and 48 in leaves in elevated [$CO_2$]. Intriguingly, the overlap between the 280 *A. thaliana* orthologues identified in our work and the complete set of genes regulated in above mentioned experiment was ten, six genes overlap between soybean roots and Arabidopsis leaves and four genes overlap between soybean and Arabidopsis roots (Fig. S2E, Table S6).

We assessed the expression profiles of the root $CO_2$-modulated genes under a variety of conditions using the Genevestigator tool [33]. The data sets of expression of Arabidopsis orthologues in different stimulus and root tissues in Genevestigator database allowed us to correlate these conditions with transcriptional responses of soybean roots to E[$CO_2$]. Expression of the Arabidopsis orthologues in response to $CO_2$ experiments selected from the Genevestigator database demonstrated that the genes are grouped in three clusters based on co-expression (Fig. S3B). Interestingly, among the 274 genes repressed in the roots of soybean plants grown under E[$CO_2$] (Table 2), 47 were also repressed in response to Fe deficiency in Arabidopsis orthologues (Fig. S3B).

### 2.4. Transcriptional responses to water deficit

Transcriptional profiling revealed 13,555 differentially expressed

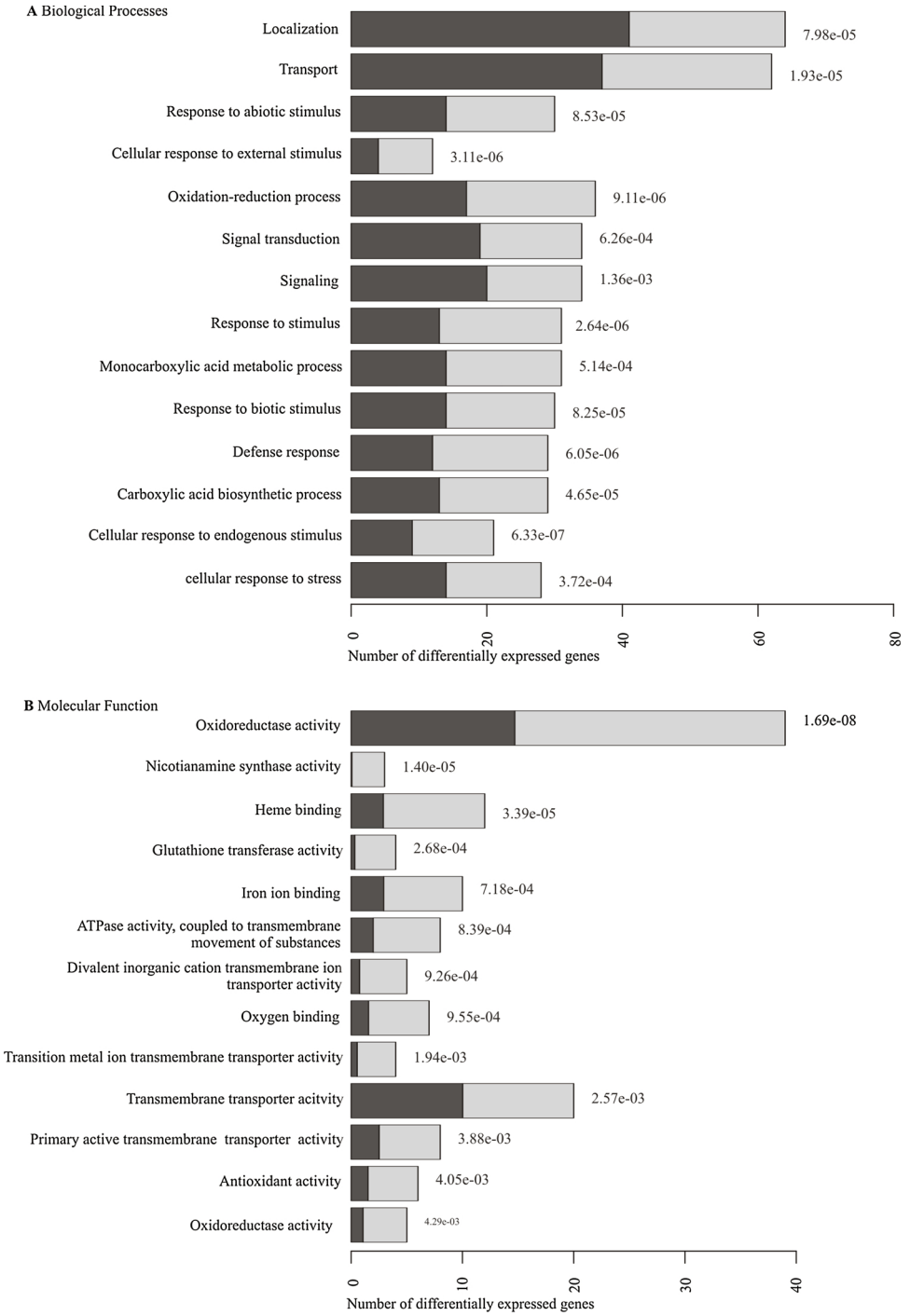


**Fig. 2.** Graphical representation of the enriched **(A)** biological processes (BP) and **(B)** molecular function (MF) from the list of differentially expressed genes (DE) between ambient and elevated [$CO_2$], observed genes, and *Arabidopsis* Gene Ontology annotations. Dark bars: expected number of DE-genes in each BP/MF; light bars: observed BP/MF enrichment; and values alongside the bars: hypergeometric test p-values. A complete list of GO categories can be found in Table S3.

genes in the roots of soybean plants submitted to 50 min of water deficit: 5193 (38.3%) were up-regulated and 8362 (61.7%) were down-regulated compared to the control (adjusted p-value $\leq$ 0.05). A log fold-change (log FC) $\geq$ 2.0 was observed in 7223 genes, 3811 (52.7%) were up-regulated and 3412 (47.3%) were down-regulated (Table 2). Among these genes, 12,143 (90.9%) were orthologues of *A. thaliana* genes (Fig. S4). The transport category includes amino acid (proline), nitrate, water, amide, and oligopeptides. Only the amino acid category has most of the genes up-regulated (57%) (Table S3).

With regard to molecular functions, the genes differentially expressed were mainly associated with transferase activity, cation binding (transcription factors, calcium ion, iron ion and carbohydrate binding), kinase activity, oxidoreductase activity, and transporter activity (Table S3, Fig. S4).

The differentially expressed genes were mapped using MapMan to generate a representative overview of the pathways affected. This analysis indicated the involvement of several genes in biotic and abiotic stress, regulation of transcription, cell division and cell cycle, development, DNA repair and synthesis, protein synthesis and amino acid activation, vesicle transport and protein targeting, protein modification and degradation, redox activity and transport (Table S8).

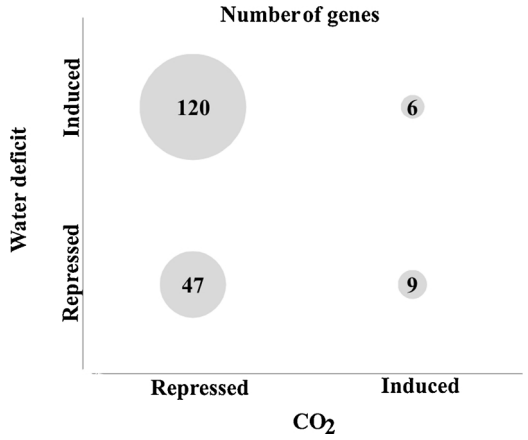


**Fig. 3.** Number of genes modulated by the combination of E[$CO_2$] and water deficit.

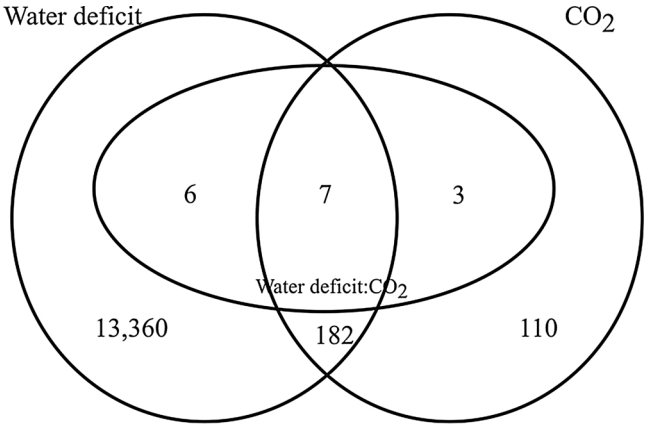


**Fig. 4.** Venn diagram illustrating the number of genes regulated by E[$CO_2$], water deficit and interaction $CO_2$:water deficit. Libraries were compared to each other to demonstrate the number of genes expressed only in one library and the ones common to different libraries.

### 2.5. Gene responses to the combination of elevated $CO_2$ and water deficit

Comparing the roots from plants grown under E[$CO_2$] with those grown only under water deficit, we observed that these two treatments share 182 genes differentially expressed (Fig. 3, Table S9). Among the 182 genes, 120 were induced in response to water deficit while being repressed in response to E[$CO_2$], and nine displayed the opposite behavior (Fig. 3, Table S9). Conversely, 47 genes were repressed and six induced in both conditions, which suggests that $CO_2$ and water deficit had an additive effect on the expression of these genes (Fig. 3, Table S9). Among the differentially expressed genes in response to water deficit, $CO_2$ reversed the expression of 41 genes, most of them associated to stress, defense, and transport categories (Table S9). These genes were analyzed using the Genevestigator tool, and clusters of co-expressed genes in response to biotic and abiotic stresses and nutrient deficiency were identified (Fig. S5).

Furthermore, it was estimated that 16 genes were differentially expressed in response to the interaction [$CO_2$]:water deficit, of which 14 were up-regulated and two were down-regulated (Table 3). The expression levels of the 16 genes in response to the combined stimulus were completely different from their expression in response to each single stimulus, thus they can be classified as an idiosyncratic interaction [34]. Among them, six genes were also modulated by water deficit alone (*AGAMOUS-LIKE 6*, *AGL6*; *AGAMOUS-LIKE 8*, *AGL8*; *BASIC PATHOGENESIS-RELATED PROTEIN 1*, *PR1*; and three genes of unknown function), and three genes were modulated by [$CO_2$] alone (*HOMOGENTISATE PHYTYLTRANSFERASE 1*, *HPT1*; UDP-*GLYCOSYLTRANSFERASE 72B1*, *GT72B1*; and *LATERAL ORGAN BOUNDARIES* 25, *LOB25*). In addition, seven differentially expressed genes overlapped among water deficit, [$CO_2$] and the interaction effect (Fig. 4). These genes include a Glyma01g33986 (prefoldin chaperone), - *FATTY ACID DESATURASE 3* (*ADS3,* Glyma08g22730), MYB transcription factor (MYB14, Glyma12g11340), a *COP1-interacting protein 7* (*CYP7*, Glyma12g35020), Glyma16g34720 (an acid phosphatase), *GLUTATHIONE S-TRANSFERASE TAU 4* (*GSTU4,* Glyma20g33951), and a gene of unknown function (Glyma04g40861) (Table 3).

We investigated the Arabidopsis orthologues of the 182 soybean genes that exhibited expression changes under both $CO_2$ and water deficit treatments, independently, using Genevestigator in a diverse set of conditions (high $CO_2$, water deficit, nutrient and other abiotic stresses). The heat map of the experiments from Genevestigator highlights several expression clusters of co-expressed genes that are grouped predominantly by treatments of the same experiment (Fig. S6).

Among the group of genes modulated in soybean roots by the interaction effect [$CO_2$]:water deficit, 11 out of 16 genes were validated by real time qPCR (see further). Eight genes with unique homologs in Arabidopsis were scrutinized in using the Genevestigator tool (seven had data available). In addition to the abiotic stresses, we also evaluated the expression in different root tissues by *in silico* analysis. Three genes (*GT72B1*, AT4G01070; acid phosphatase member AT2G38600;

**Table 3**
Expression data of genes modulated in response to water deficit, E[$CO_2$], and interaction $CO_2$:water deficit.

| Gene | ATCode | Gene Symbol | Description | $CO_2$ | | Water deficit | | $CO_2$:Water deficit Interaction | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | logFC | FDR | logFC | FDR | logFC | FDR |
| Glyma01g33070 | AT2G18950 | HPT | Homogentisate Phytyltransferase 1 | −1.265 | 0.0000 | −0.094 | 0.8531 | 1.214 | 0.0239 |
| Glyma01g33986 | AT1 G03760 | – | Prefoldin Chaperone | 4.951 | 0.0000 | 2.918 | 0.0045 | −7.632 | 0.0000 |
| Glyma03g26980 | AT4G01070 | GT72B1 | UDP-Glycosyltransferase | −2.967 | 0.0000 | 0.860 | 0.1176 | 2.927 | 0.0166 |
| Glyma04g29490 | – | – | – | −0.632 | 0.7102 | −1.300 | 0.0003 | 2.248 | 0.0028 |
| Glyma04g31847 | AT2G45650 | AGL6 | AGAMOUS-like 6 | −0.140 | 1.0000 | −7.537 | 0.0000 | 8.526 | 0.0000 |
| Glyma04g40861 | – | – | – | −1.150 | 0.0000 | 3.011 | 0.0000 | 1.357 | 0.0017 |
| Glyma07g11075 | – | – | – | −2.318 | 0.2905 | −2.818 | 0.0056 | 5.134 | 0.0485 |
| Glyma08g22730 | AT3G15850 | ADS3 | Fatty Acid Desaturase 5 | −4.113 | 0.0061 | 1.258 | 0.0343 | 4.885 | 0.0126 |
| Glyma12g11340 | AT2G31180 | MYB14 | MYB14 transcription factor | −1.605 | 0.0007 | 6.108 | 0.0000 | 1.776 | 0.0120 |
| Glyma12g35020 | AT4G27430 | CIP7 | COP1-interacting protein 7 | 0.761 | 0.0378 | 0.747 | 0.0017 | −1.395 | 0.0079 |
| Glyma15g06790 | AT2G14580 | | Basic pathogenesis-related protein 1 | −0.601 | 0.2741 | −0.832 | 0.0007 | 1.454 | 0.0079 |
| Glyma16g34720 | AT2G38600 | – | Acid Phosphatase | −1.128 | 0.0000 | 1.161 | 0.0000 | 1.299 | 0.0012 |
| Glyma17g08890 | AT5G60910 | AGL8 | AGAMOUS-like 8 | 0.674 | 0.3967 | −2.274 | 0.0000 | 1.964 | 0.0028 |
| Glyma19g12650 | AT3G27650 | LBD25 | LOB domain-containing protein 25 | −2.092 | 0.0161 | −0.835 | 0.1837 | 3.049 | 0.0357 |
| Glyma20g03850 | – | – | – | −0.858 | 0.1372 | −1.033 | 0.0009 | 1.715 | 0.0239 |
| Glyma20g33951 | AT2G29460 | GSTU4 | Glutathione S-transferase Tau 4 | −2.612 | 0.0001 | 1.374 | 0.0014 | 2.720 | 0.0239 |

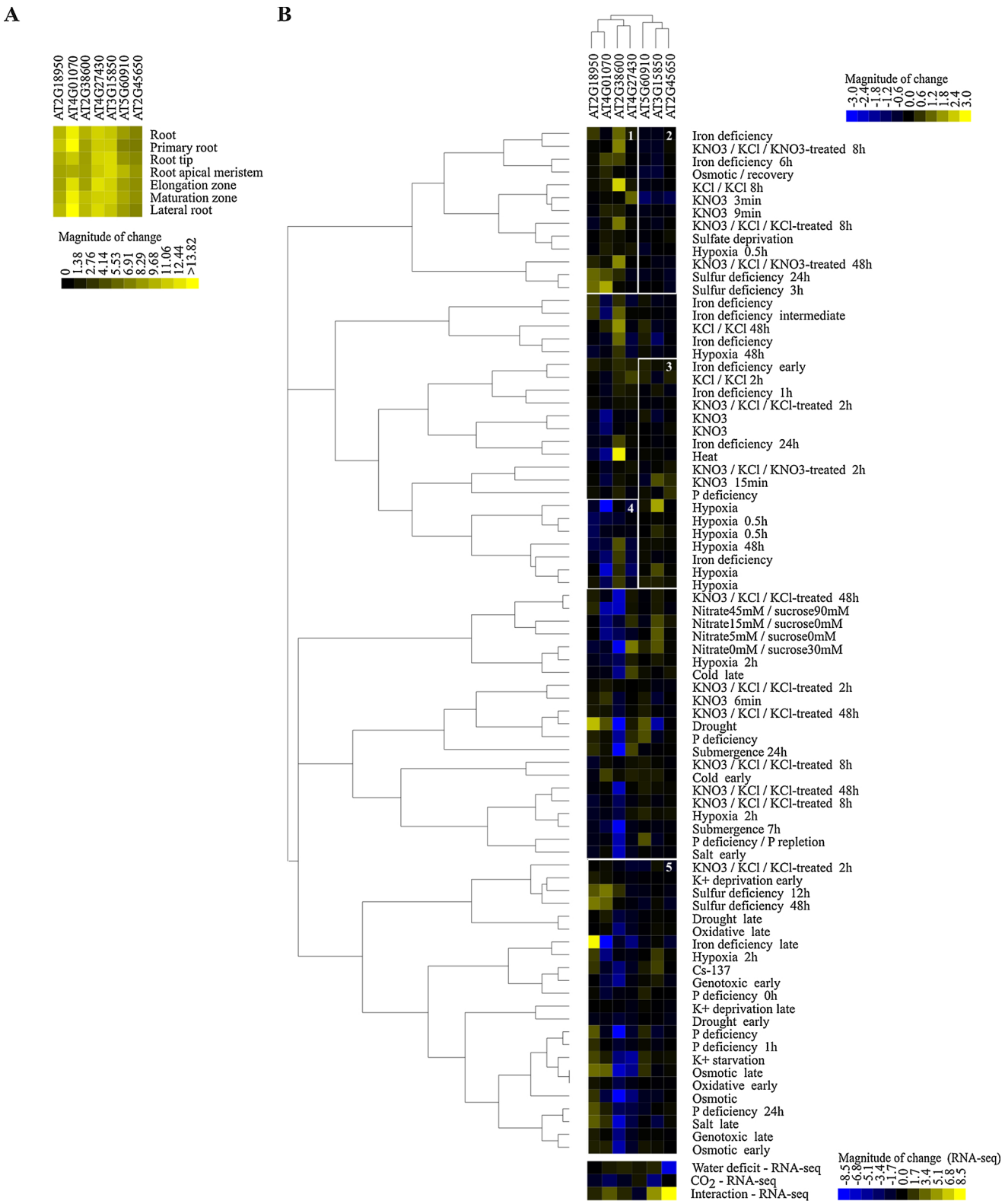


**Fig. 5.** Heat map illustrating the expression of Arabidopsis orthologues of soybean root genes regulated by the interaction [$CO_2$]:water deficit. Arabidopsis orthologous of the soybean genes were analyzed in Genevestigator to evaluate responses to different tissues and abiotic and nutrient stresses. Data were available for seven out of 11 genes (AT2G18950-*HPT*, AT4G01070-*GT72B1*, AT2G38600-acid phosphatase, AT4G27430-*CYP7*, AT3G15850-*ADS3*, AT2G45650 -*AGL6*, AT5G60910-*AGL8*). **(A)** Different root tissues. **(B)** Abiotic and nutrient stresses. Only DNA microarrays experiments performed in root tissues were selected for this analysis (root filter). When we analyzed gene expression under nutrient deficiency, four main clusters of expression were outlined: Groups 1, and 3 include genes induced in response to nutrient deficiency conditions ($KNO_3$, nitrate, iron, and potassium and hypoxia); and Group 2 and 4 are composed of genes down-regulated in response to nutrient deficiency conditions ($KNO_3$, nitrate, iron, and potassium and hypoxia). The magnitude of change in **(A)** and **(B)** are indicated at the botton and at the top of the figures, respectively. The data of the RNA-seq of the present work and the respective magnitude of change are indicated at the bottom of figure **(B)**. Down-regulated genes are depicted in blue and up-regulated genes in yellow. The intensities of the colors increase with increasing expression differences (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

and *CYP7,* AT4G27430*)* were mainly expressed in lateral roots, specifically in the maturation and elongation zones (*GT72B1* and acid phosphatase) or in the root apical meristem (*CYP7*), whereas *HPT1(*AT2G18950), *ADS3 (*AT3G15850), *AGL6 (*AT2G45650), and *AGL8 (*AT5G60910) had a low expression throughout the whole root (Fig. 5A). *GT72B1*, *CYP7*, acid phosphatase, *AGL6*, and *AGL8* were also expressed in soybean roots [35], Soybean eFP Browser at bar.utoronto.ca [36]. The results from abiotic and nutrient conditions were biclustered (genes and treatments), and five main expression groups were apparent: cluster 1 and 2 mainly comprised genes up- and down-regulated by nutrient deficiency (Fe, $KNO_3$, P, KCl, Cs, and sulfate), respectively; clusters 3 were composed of genes up-regulated by nutrient deficiency and abiotic stresses; cluster 5 included genes repressed by nutrient deficiency and abiotic stresses; and cluster 4 mainly comprised genes down-regulated by hypoxia (Fig. 5B). In our experiments, the expression of genes *HPT*, *GT72B1*, and Glyma16g34720 -acid phosphatase were induced in roots by the interaction $CO_2$:water deficit, showing a similar pattern observed in cluster 1; and these genes were repressed by E[$CO_2$], showing a similar pattern observed in cluster 4.

### 2.6. Validation of RNA-seq results by quantitative RT-qPCR

To validate RNA-seq data, the two biological samples assayed by RNA-seq were used as source material for the qPCR analysis. We also included an additional biological replicate to the RNA-seq validation to increase the robustness of our data. Thirty-two genes differentially modulated in response to E[$CO_2$] and/or water deficit and 16 genes with expression affected by the interaction effect $CO_2$:water deficit were chosen for validation by qPCR. Primer pairs were design for each gene (Table S10). The expression pattern was confirmed for 11 of the 13 genes responsive to $CO_2$ and 18 out of the 19 genes responsive to both water deficit and $CO_2$, 29 in total (Fig. S8).

## 3. Discussion

### 3.1. Physiological responses to E[$CO_2$] and water deficit

Studies with soybean [12,37] and $C_3$ plants in general [10] have demonstrated that photosynthesis is typically elevated by about 20% under E[$CO_2$]. The photosynthesis rate observed in our experiment (Fig. 1A) was lower than what is usually observed in field experiments [12,37,38]. This is probably due to the reduced light level and lower temperatures in the Open Top Chambers (OTCs) (600 μmol $m^{-2}s^{-1}$ and 20 °C), but can also be attributed to our experimental design. It was also observed in this experiment, that soybean plants grown under E[$CO_2$] had a 103% increase in photosynthesis (Fig. 1A, T0), a much higher induction that is usually observed in potted or field experiments. This might be related to the fact that soybean plants were grown under hydroponic system, since the experimental conditions have a substantial influence on the percentage of change under E[$CO_2$] [8]. Interestingly, the photosynthesis rates under E[$CO_2$] did not reduced after 50 min despite the reduction of stomatal conductance. Although the commonly observed correlation between photosynthesis and stomatal conductance can be disrupted in certain conditions [39], it is unclear why this happened in our experiment.

With a double of photosynthesis, plants under E[$CO_2$] had the biomass, plant height, and leaf area increased (Fig. 1G–I). Root proliferation and elongation are typically positively affected by increases in the [$CO_2$] concentration [13,21,40–42]. In the present study, soybean root length remained constant, although the root dry biomass was higher at E[$CO_2$] (Fig. 1J, E), probably due to increase in the number of lateral roots and lateral roots length [43,44]. It is important to mention that these differences in growth rate might have little affected on gene expression comparison analysis since all plants (grown under E[$CO_2$] and A[$CO_2$]) were at the same developmental stage.

The reduction in stomatal conductance observed at E[$CO_2$] led to an increase in water use efficiency, which promoted the maintenance of photosynthetic rates and fresh weight of leaves in this treatment up to 25 min of water deficit (Fig. 1A–D). According to Wall et al. [45], Robredo et al. [19], Leakey et al. [46], Ainsworth and Long [10], and Long et al. [9], lower stomatal conductance improves water-use efficiency, which alleviates the effects caused by water deficit. In our experiment, the improvement in water use efficiency led to a higher RWC under E[$CO_2$] (Fig. 1F). The benefit caused by this response can be observed especially after 50 min of water stress, where the reduction in the fresh weight of leaves and roots under E[$CO_2$] was lower than under A[$CO_2$] (Fig. 1C, E). Altogether, these physiological responses suggest that E[$CO_2$] buffers the adverse effects of water deficit by maintaining the water content in the plant.

### 3.2. Responses to E[$CO_2$] at a molecular level

Among the methods used to transcriptionally profile plant responses to global climate change, DNA microarray analysis has been the most common [12]. Using this technique, many authors observed that 0.3–5% of genes appear to be differentially expressed between ambient and elevated $CO_2$ [12,29,47–54].

RNA-seq provides greater sensitivity than DNA microarrays. It does not suffer from hybridization-based limitations such as background noise and saturation, or with probe set issues such as incorrect annotation and isoform coverage. RNA-seq is more sensitive in detecting genes with very low expression and more accurate in detecting expression of extremely abundant genes [55]. Once the largest fold changes in transcript abundance are typically twofold at E[$CO_2$] [12], we chose add sequencing depth to the biological replicates, which improved estimation accuracy mostly for low expression genes [56]. This strategy was proved accurate, considering our qPCR validation data.

Using mRNA-seq, we identified 302 $CO_2$-modulated genes, approximately ˜0.5% of the *G. max* roots transcriptome (Fig. 4, Table S2). Thus, similar to other studies in leaves, we observed that soybean roots exposed to E[$CO_2$] displayed a few transcriptional changes. Surprisingly, the number of down-regulated genes (274) in response to E[$CO_2$] in roots was much higher than the number of up-regulated genes (28) (Table 2). These results contrast with observations made in the leaves of soybean and other species at E[$CO_2$], in which the number of up-regulated genes was greater than the number of down-regulated genes [12,29–31]. The high number of down-regulated genes in roots compared to leaves under E[$CO_2$] observed in this study highlight the metabolic differences between them. In addition, methodological differences such as growth system and fumigation method may have contributed to differences between the results of the present study and previous studies. Leaves are the first organ that directly senses E[$CO_2$] through an increase in photosynthesis [11]. Therefore, it is expected that E[$CO_2$] would up-regulate a higher number of genes in the leaves compared to roots [29,31]. In spite of this difference and the small overlap among the genes regulated in roots and leaves by E[$CO_2$], they share functional similarities. For example, the transport, secondary metabolism/defense, and stress categories that were modified in roots in our study were also affected by E[$CO_2$] in leaves [12,29,31]. When *Eucalyptus grandis* roots colonized by different *Pisolithus* sp. isolates were grown under conditions of ambient (400 ppm) and elevated (650 ppm) $CO_2$, a dramatic shift in the root transcriptomic profile was observed [57]. Although the difference in species and the presence of a biotic effect restrains further comparisons with our results, Plett et al. [57] observed that the number of $CO_2$-modulated transcripts is equally divided between up- and down-regulated genes.

In the present study, transport-associated transcripts were mainly mineral nutrient transporters (Table S2, S3), and several of these transcripts are related to the transport and cellular response to iron. Recently, a meta-analysis of crops grown under field conditions at ambient and E[$CO_2$] revealed that $C_3$ grains and legumes have lower concentrations of zinc and iron when grown at E[$CO_2$] [58,58]. In

addition, Jauregui and collaborators identified lower levels of Fe, Zn, Mo, Mn, Cu and Ni in *A. thaliana* plants grown under E[$CO_2$] [32]. It has been suggested that the lower concentration of leaf minerals might be a side effect of the decrease in transpiration rate which consequently reduces xylem flux [60,61]. Other authors propose that the mineral composition depletion is result of the dilution effect, since plants grown under E[$CO_2$] produce more carbohydrates [62,63]. However, our data in soybean and the work of Jauregui and collaborators [32] with *A. thaliana* suggest that changes in the expression regulation of genes encoding for transporters could be the major factor for the lower concentration of leaf minerals grown under E[$CO_2$]. In Arabidopsis, the iron uptake system controlled by the root is highly dependent on the expression of *FRO2*, a ferric-chelate reductase [64]. *FRO2* was repressed in the present study under E[$CO_2$]. Furthermore, *FER1* was also down-regulated, and it is known that the majority of iron is stored in chloroplast ferritin proteins [65]. A variety of transporters has been demonstrated to participate in the iron translocation process from root epidermal cells to leaf cells, including *YELLOW STRIPE-LIKE* (*YSL*). YSL has been suggested to transport metals complexed with nicotianamine (NA), which is essential for iron distribution in plants [66] and is synthesized by nicotianamine synthase (NAS) [67]. *YSL* and *NAS1*, *2,* and *3* were down-regulated in the present study, and *NAS1* and *NAS3* were down-regulated in Arabidopsis leaves under E[$CO_2$] [30]. In addition to the iron-responsive genes, *ZIP2* and *ZIP10*, involved in manganese/zinc and iron transmembrane transport [68], are also down-regulated by E[$CO_2$] in soybean roots. Genevestigator analysis suggested that Fe deficiency affects the same group of genes (Fig. S3A), reinforcing the idea that cultivation under E[$CO_2$] affects nutrient transport in soybean roots. The repression of iron-mediating genes, and also other mineral nutrient transporters, in roots may result in lower mineral concentrations in the leaves and seeds of soybeans grown at E[$CO_2$] [59]. However, additional experiments are necessary to better understand the effect of E[$CO_2$] on the mineral transport of soybean roots.

Another gene category affected by E[$CO_2$] is associated with secondary metabolism processes (Table S3). E[$CO_2$] exposure in soybean repressed phenylalanine, phenylpropanoid, and flavonoid biosynthesis pathways, as the expression of *PAL1*, *CAD6*, and five naringenin-chalcone synthases (CHS – *TT4*) was repressed (Table S2). These observed changes could alter the interaction between plant and microorganisms, as the rhizosphere flavonoids perform a wide range of functions, including defense against pathogens and disease. It might interfere with the root nodulation process which could have dramatic effects on soybean yields. Moreover, they participate in the regulation of root growth and possess metal chelator activity, which makes iron and phosphate available to the plant (reviewed by Cesco et al. [69]). Consistently, the down-regulation of genes that mediate flavonoid biosynthesis were also observed in the leaves of Arabidopsis grown under E[$CO_2$] [31]. Casteel et al. [70] observed that transcripts from genes that regulate flavonoid/phenylpropanoid production were down-regulated in the leaves of soybean plants exposed to E[$CO_2$], leaving plants more vulnerable to herbivores. Furthermore, the fact that phenylpropanoid metabolism has been down-regulated suggests that a lignification process might be affected by E[$CO_2$], which has been observed in soybean leaves by Ainsworth et al. [29].

E[$CO_2$] also appears to affect the antioxidant activity in roots (Fig. 2B, Table S3). Several studies have reported that E[$CO_2$] increases the synthesis and activities of antioxidants that tend to alleviate the problems caused by oxidative stresses [25,42]. However, we identified ten different soybean genes encoding putative peroxidases and one *FER1* gene [27,28] that were down-regulated under E[$CO_2$]. In addition, genes from the glutathione pathway (*GSTU4*, *GSTU7*, *GSTU19,* and *GSTU8*) were down-regulated. Similar to peroxidases, glutathione participates in cell detoxification [71]. Although antioxidant-related genes were repressed in soybean roots, they were induced in the leaves of plants grown under E[$CO_2$] [12,31,72], probably because leaves require these proteins to respond to increased Reactive Oxygen Species (ROS) production by enhanced photosynthesis [73] at E[$CO_2$], which did not occur in roots.

Transcription factors (TFs) represented 10.6% of the differentially expressed genes in response to E[$CO_2$] (Table S2). Other $CO_2$ studies have also reported a high number of genes encoding TFs (˜1.5–7%) [29–3151]. In our study, five TFs were up-regulated and 25 down-regulated (Table S2). They predominantly mediate stress and defense (e.g., ERF1B, ERF15, RAP2.6, SCL14, HB12, MYB15, HSFB3, WRKY51, WRKY40) and development (e.g., GLABROUS, ATBS1, BLH11, CPC, WRKY75). This corroborates the hypotheses raised above by demonstrating differential expression of defense-related genes. Li et al. [30] also observed the repression of *WRKY75* in leaves under E[$CO_2$]. Other TFs that overlapped between Li et al. [30] and our experiment (ERF1B, HB12, CPC) were up-regulated in leaves. In Arabidopsis, *wrky75* mutants and *WRKY75* RNAi plants exhibited ectopic root hair development [74]. Rishmawi et al. [74] demonstrated that *WRKY75* is expressed in the pericycle and vascular tissue and that the *WRKY75* RNA or protein moves into the epidermis. The authors claim that WRKY75 regulates root hair patterning by repressing CPC and TRY. Our results indicate that repression of *WRKY75* in plants under E[$CO_2$] promote root hair growth. This result corroborates the proposal that WRKY75 mediates various environmental responses and that its down-regulation provides an easy and valuable way to modulate root hair number.

### 3.3. Transcriptome profiles after short-term water deficit

The hydroponic system does not represent an actual field condition [75]. However, it greatly facilitates rapid and easy root sampling and eliminates combinatorial eff ;ects brought by heat and other environmental factors, commonly experienced in field-grown plants [75]. The hydroponic growth system also permits acute instantaneous water deficit treatments, by removing the plants out of the solution. This strategy has been successfully used before in assays of water deficit stress in soybean in several works [18,76–79], and this was also the strategy that was used in this study. The abrupt water deficit in the hydroponic system causes severe consequences to soybean plants, impeding the continuous acclimation process that occurs in the pot-based system or field [76,77,80]. Therefore, a hydroponic system allows tracking the response to water deficit at early stages, highlighting the key signaling players in the signaling cascade.

Comparing our RNA-seq results to the ones obtained by Rodrigues et al. [18] and Neto et al. [81], in which Embrapa 48 cultivar were also grown in the hydroponic system and submitted to a short-term water deficit stress, similar enriched GO categories were found between the up-regulated genes in roots. The common categories are signaling, response to stress (related to water deficit), response to biotic stimulus, senescence, response to nitrogen compounds, and secondary metabolic processes.

In our study, we identified early signaling players in soybean roots in response to 50 min of the water deficit. The signaling category comprises receptors for water deficit sensing such as members of the Mitogen-Activated Protein Kinases (MAPK) pathway and components of $Ca^{2+}$-dependent signaling pathway. It is uncommon to find reports from gene expression studies showing significant differences in the expression of *MAPK* genes in roots under water deficit. This lack of data may be related to a very rapid initiation of *MAPK* gene expression in roots upon the occurrence of the water deficit, followed by their fast return to initial levels of expression [82,83]. Genes for components of $Ca^{2+}$-dependent signaling pathway identified include homologous genes of calcium-dependent protein kinases (CDPKs), calcineurin B-interacting protein kinases (CIPKs), calmodulin-related calcium sensor proteins (CML) and protein phosphatases class 2C (PP2C) (Table S7). These genes may be up- or down-regulated in root response to water deficit [84–87]. An important water deficit signaling in roots is regulated by abscisic acid (ABA) [17], which is involved in the early

response to water deficit stress. Among the ABA-dependent TFs, *ABF2* and *ABI5* were down-regulated and *MYC2* and *MYB2* were up-regulated (Table S7). In addition, rapid induction of water deficit stress induces ethylene production [88,89]. Examples of genes identified in the present study induced by ethylene are *ERF1* and *DREB1 A* (Table S7).

### 3.4. Transcriptome profiles after E[$CO_2$] and water deficit combination

Our physiological data revealed that E[$CO_2$] mitigates the negative effects of water deficit. We also presented evidence of this mitigation on a gene expression level. Among the genes that were repressed in E[$CO_2$] and induced by water deficit, 37 were repressed when both treatments were applied at the same time (Table S9). In this case, E[$CO_2$] reverted the expression of water deficit-induced genes mainly related to stress, defense, transport and nutrient deficiency (Table S9, Fig. S6). In addition, some of these genes participate in development. Interestingly, two key genes (WRKY75 and LOX1) that govern root architecture are induced by water deficit, repressed by E[$CO_2$] singly and in combination with water deficit. Both WRKY75, a main regulator of root hairs, and LOX1 (*LIPOXYGENASE 1*), a putative regulator of the emergence of lateral roots [90], probably promote root growth as an adaptive response to water deficit [91].

The effects of the combination of E[$CO_2$] and water deficit might be characterized as: (i) the addition of single stress responses (additivity), (ii) more than the sum of the individual stresses (synergy), (iii) completely distinct from single stress responses (idiosyncrasy), or (iv) something close to one of the combined stresses (dominance) [34]. In addition to the 182 genes which had a $CO_2$ and water deficit additive effect on their expression, the combination of both treatments uniquely affected the expression of 11 genes (Table 3). This effect, classified as an idiosyncratic interaction in our work, resulted in nine up-regulated and two down-regulated genes (Table 3). The up-regulated genes include *GT72B1*, *HPT1*, *ADS3*, *AGL6,* and *AGL8* (Table 3). *GT72B1* is essential for cell wall lignification in Arabidopsis [92]. HPT1 is the first committed enzyme in tocopherol biosynthesis [93]. Tocopherols are lipid-soluble antioxidants that regulate defense responses by modulating levels of lipid peroxidation products [94]. Further, *ADS3* (*FAD5*) encodes the plastidic palmitoyl-monogalactosyldiacylglycerol Δ7 desaturase [95]. Arabidopsis *fad5* plants present a drastic reduction on the chlorophyll content that may be due to a reduced level of unsaturated MGDG adversely affecting photosystem. Increased unsaturated fatty acid contents are believed to maintain the fluidity and stability of cellular membranes during plant adaptation to stresses [96]. Therefore, among the genes identified with known function, two of them are associated to membrane protection and fluidity (*HPT1* and *ADS3*). AGL6 mediates the transition to flowering and participates in lateral organ development [97,98]. AGL8 acts during fruit development, floral meristem identity specification, shoot maturation, and during floral transition [99–106]. A comprehensive interaction analysis indicated that AGL6 interacts with AGL8 [107]. One of the genes repressed by the interaction effect encodes a prefolding chaperone, which is associated with protein folding, flowering, and photoperiodism. An additional gene, *CIP7*, positively regulates anthocyanin accumulation [108] (Table 3). However, more studies are necessary to understand the roles of *AGL6*, *AGL8*, prefolding chaperone, and *CIP7* in roots and under water deficit. We next aim to characterize these genes in soybean roots by assessing their effects after overexpression and silencing.

## 4. Conclusions

This is the first study that evaluates the physiological and molecular aspects of soybean roots grown under E[$CO_2$], singly and in combination with water deficit. E[$CO_2$] increased photosynthesis and biomass and, at the molecular level, predominantly decreased gene expression related to transport, secondary metabolism, antioxidant activity and transcription factors. Water deficit also decreased expression of genes related to transport, oxidative stress and secondary metabolism, however induced hormone signaling pathways and defense, and differentially altered many transcription factors. When applied simultaneously, E[$CO_2$] reverted the expression of water deficit-induced genes mainly related to stress, defense, transport and nutrient deficiency. In addition, the response of soybean roots grown under E[$CO_2$] to short-term water deficit include the expression of genes that are not activated by the two treatments alone suggests that the buffering effect in water deficit observed of soybean plants under E[$CO_2$] implies an emergent response that is triggered only under these specific conditions. Although our data cannot be extrapolated to either field condition or to a general condition of stress, the finding that a mechanism is associated to membrane protection and stability (*HPT1* and *ADS3*) and reproductive development (*AGL6* and *AGL8*) under water deficit combined with E[$CO_2$] sets future interesting targets for research on how roots may be involved in the responses of soybean to climate changes.

## 5. Material and methods

### 5.1. Plant material and growth conditions

Soybean seeds from a drought-tolerant cultivar (*Glycine max* cv. Embrapa 48; [109]) were provided by EMBRAPA Soja. They were germinated on filter paper for seven days in a growth chamber (TE-3911, Tecnal, Brazil) at 25 ± 2 °C, 100% air humidity, and a 16/8 h light/dark cycle. The seedlings were transferred to 36 L boxes containing 50% Hoagland's solution [110], in which a hydroponic system was established. The boxes with 30 seedlings each were distributed through four open-top chambers (OTCs) 1.5 m in diameter and 1.68 m in height (two boxes per chamber) localized inside a greenhouse. The Hoagland's solution was continuously aerated and replaced weekly. Two OTCs, containing two boxes with 30 plants each (60 plants in total for each OTC), were kept under E[$CO_2$] (approximately 800 ppm) while two other chambers, in the same total number of plants, were provided with ambient $CO_2$ (A[$CO_2$]) (approximately 400 ppm) throughout the duration of the experiment (from seedling to plants at V3/V4 stage). The temperature, humidity, and $CO_2$ concentration of each chamber were recorded every 30 min using a Remote Integrated Control System (RICS). A hydroponic system was used because it facilitates rapid and easy root sampling, and is less influenced by external factors. Additionally, this growth system permits acute water deficit treatments by removing the plants from the solution. The experiment was conducted for 24 days (September 23 to October 16, 2013) in a greenhouse at the Laboratório de Fisiologia Ecológica de Plantas (LAFIECO), University of São Paulo, São Paulo, Brazil, under natural environmental conditions. To normalize microenvironmental differences due to the position of the box in different OTCs, the boxes were changed weekly between chambers having the same $CO_2$ concentration. During the experimental period, the air temperature inside the OTCs ranged from 17.4 to 28.3 °C, and the relative air humidity (RH%) ranged from 48.3 to 83.2% (Fig. S9). The plants were allowed to grow until the V3/V4 stage [111]. After this period, water deficit was applied by randomly removing plants out of the hydroponic solution exposing the roots to ambient- or elevated-air for 0 (T0; control), 25 (T25), or 50 (T50) min [18]. Sampling for RNA-seq analysis across ambient versus elevated [$CO_2$] treatments was collected alternately in a total time of 2 h, to avoid circadian fluctuations in gene expression. During this period, the air temperature was 20 °C, and the relative air humidity was 80%. For each water deficit time point in the elevated and ambient $CO_2$ treatments, roots from 15 plants (five from each box - biological triplicates) were collected and immediately frozen in liquid nitrogen.

### 5.2. Leaf gas exchange, growth, and biomass

The net leaf $CO_2$ assimilation rate ($A$) and stomatal conductance ($g_s$) were measured in the youngest fully expanded leaves from 10:00 to

12:00 h using a portable open gas-exchange system incorporating infrared $CO_2$ and water vapor analyzers (LI-6400 XT; LI-COR, Lincoln, NE, USA). During the measurements, the light intensity was kept at 600 μmol $m^{-2}s^{-1}$ and the leaf temperature at 20 °C, according to natural conditions. The $CO_2$ concentration used for the gas exchange analysis was the same for both growth conditions (400 or 800 μmol $CO_2$ $mol^{-1}$ air). The plants were measured at times 0, 25, and 50 min after water deficit (T0, T25, T50). For each water deficit time point, three biological replicates (pool of five plants from each box) were analyzed.

Plant height data were obtained from the root-shoot transition to the tip of the youngest leaf. Root length was determined by measuring from the root-shoot transition to the tip of the longest root. The fresh weight of the leaves, stems, and roots were determined immediately after each water deficit period. For calculating the dry biomass, each organ was dried in an oven at 60 °C until a constant weight was obtained. The leaf area was quantified by scanning the leaves and processing the images using the software Image-Pro Plus version 6.3. All these data were collected from plants from the same conditions of plants used for RNA-seq.

### 5.3. Leaf relative water content (RWC)

The RWC was measured according to Barrs and Weatherly [112]. Immediately after sampling, leaf discs with a 1 cm diameter were weighed and immersed overnight in distilled water at 4 °C. Next, they were blotted dry and weighed prior to oven-drying at 60 °C for 24 h. The dry weight of the leaf discs was then determined. The leaf relative water content was calculated using the following formula: RWC = ((FW – DW)/ (TW-DW)) x 100, where FW is the fresh weight, DW is the dry weight, and TW is the turgid weight (weight after the leaf was stored in distilled water overnight).

### 5.4. Statistical analysis of physiological data

The biomass, height, and leaf area were analyzed using a one-way ANOVA, (the time out the hydroponic solution was not considered as a factor because it is not suppose to affect growth), followed by Tukey's honest significant pair-wise difference post-hoc tests. The variables sampled along time (0, 25, and 50 min, n = 3 each) were analyzed using a two-way ANOVA followed by Tukey's honest significant pair-wise difference post-hoc tests, where the $CO_2$ treatment and time of water deficit were fixed factors. All statistical analyses of the physiological parameters were performed using the software JMP version 5.2.

### 5.5. RNA extraction and sample preparation

After the fresh weight was measured, the roots were immediately frozen in liquid $N_2$ and stored at −80 °C. The whole process took less than two minutes to be completed. The samples were ground to a fine powder using a pestle and a mortar. Total RNA was extracted using Trizol reagent (Invitrogen) according to the manufacturer's instructions. Samples from each plant were extracted separately, and equimolar amounts of total RNA from five plants were pooled and clarified with the Plant RNAeasy kit (Qiagen) with an on-column DNase digestion according to the manufacturer's instructions. The RNA quantity and quality were assessed using a Bioanalyzer Chip RNA 7500 series II (Agilent). The samples used in transcriptome sequencing included A[$CO_2$]-T0 (ambient [$CO_2$] without water deficit), E[$CO_2$]-T0 (elevated [$CO_2$] without water deficit), A[$CO_2$]-T50 (ambient [$CO_2$] 50 min of water deficit), and E[$CO_2$]-T50 (elevated [$CO_2$] 50 min of water deficit). Two biological replicates (pool of five of one of the boxes from each of the two OTCs) of each treatment were sequenced. The ENCODE consortium in Standards, Guidelines and Best Practices for RNA-Seq v1.0 [113] describes that RNA-seq experiments should be performed with two or more biological replicates and that the ability to detect reliably low copy number transcripts depends upon the depth of sequencing and on a sufficiently complex library. Library preparation and massive parallel sequencing were performed by Eurofins MWG Operon (Huntsville, AL). The sequencing libraries were prepared using the NEBNext Ultra Directional RNA Library Prep Kit (New England Biolabs, MA). Briefly, poly-A-containing mRNA was isolated from 5 μg total RNA using two rounds of purification with poly-T oligo-attached magnetic beads and fragmented with sonication. First-strand cDNA was generated using reverse transcriptase and random primers. Following second-strand cDNA synthesis and adaptor ligation, 300-bp-cDNA fragments were isolated using gel electrophoresis and amplified by polymerase chain reaction (PCR). The products were loaded onto an Illumina HiSeq 2000 instrument and subjected to 200 cycles of paired-end (2 × 100 bp) sequencing. The processing of fluorescent images into sequences, base-calling, and quality value calculations were performed using the default setting of Illumina data processing pipeline (version 1.8).

### 5.6. Reads mapping and differential gene expression

Raw reads were filtered by removing low-quality reads containing more than 30% bases with Q < 20 (99% accuracy for base calls). After trimming low-quality bases from the 5′ and 3′ ends of the remaining reads, the resulting high-quality reads were aligned against the *Glycine max* genome v.1.1 (https://phytozome.org) using the BWA package [114]. Differential gene expression was estimated and tested with the edgeR software package in R-bioconductor 2.15 [115]. The count data were normalized to the total number of counts while accounting for the variance and the mean of the biological replicates. We assessed the significance of the two main sources of variability affecting gene expression, $CO_2$, water deficit, and the interaction between them, by the adjustment of overdispersed Poisson regression models with $CO_2$ and water deficit as fixed effects. Multiple testing type I errors were adjusted with a false discovery rate [116].

### 5.7. Functional annotation

The differentially expressed genes were subjected to BLAST using the program blastx with the TAIR 9 protein database (e-value cutoff of $10^{-5}$) and further classified into categories according to the Gene Ontology (GO) classification system. RNA-seq data can be accessed at the NCBI bioprojects under the accession number PRJNA295411. To identify relevant molecular mechanisms potentially associated with the response to plant water deficit stress and E[$CO_2$], Gene Set Enrichment Analysis (GSEA) was performed [117]. A gene set was defined as all genes differentially expressed, with annotations according to *Arabidopsis*, that share the same ontology based on the results from the GO database. The GSEA method identified biological processes (BP), molecular functions (MF), and cellular components (CC) that were over-represented among the list of differentially expressed genes. The over-representation was assessed with a statistical score based on a hypergeometric test with p-values ≤ 0.005. All of the differentially expressed genes were also subjected to KOBAS analysis (http://kobas.cbi.pku.edu.cn/home.do), and significant pathways were selected at p-values ≤ 0.05. KOBAS is a web server that annotates a set of genes with putative pathways by allowing cross-species sequence similarity mapping [118]. Moreover, the STRING database (http://string-db.org/) was used to assemble information about both known and predicted protein-protein associations. STRING is a web resource that assesses and integrates protein–protein interactions, including physical and functional associations, with other resources [119]. These results were based on numerous sources, including experimental repositories, computational prediction methods, and public text collections [119]. Differentially expressed genes were further queried against the nutrient and abiotic stress Arabidopsis-related transcripts in GENEVESTIGATOR (https://www.genevestigator.com/gv/plant.jsp). The observed data sets were subjected to hierarchical average linkage (Spearman Rank Correlation)

bi-clustering (gene and array) using Cluster/Treeview [120]. In addition, the differentially expressed genes were also functionally analyzed using MapMan software, which is a user-driven tool that displays large genomic datasets onto diagrams of metabolic pathways or other processes [121].

### 5.8. Quantitative real time PCR (qPCR) analysis

To validate RNA-seq expression at 50 min and also to verify the expression of selected genes after 25 min of water deficit, qPCR was conducted. Three independent biological samples for each experimental condition were evaluated using technical triplicates. Two micrograms of total RNA from each sample were reverse transcribed using 0.5 μL of random primers (C1181, Promega) and Superscript III reverse transcriptase (Invitrogen) according to the manufacturer's instructions. The primers were designed using the Primer 3 plus software [122]. PCRs were performed using the 7500 Fast Real-Time PCR detection system (Applied Biosystems), and SYBR Green was used to monitor dsDNA synthesis. A ROX passive reference dye was used to provide a constant fluorescent signal for sample background correction throughout the qPCR assay. The fluorescence accumulation data from triplicate qPCR reactions for each sample were used to fit four-parameter sigmoid curves to represent each amplification curve using the qpcR library [123] and the R statistical package version 3.1.2 [124]. The quantification cycle, identified by a characteristic point or crossing point, Cp, was determined for each amplification by the maximum of the first derivative of the fitted sigmoid curve. The efficiency of each amplification reaction was calculated as the ratio between the fluorescence of the quantification cycle and the fluorescence of the cycle immediately preceding that. The estimated efficiency of each gene was obtained by averaging all efficiencies calculated for that gene. The reference genes *FBOX* and *ACT11* [125] were used to normalize between the different amplified samples and were previously selected with the geNorm method [126]. We compared the means of normalized gene expression values among groups (A[$CO_2$]-T0, A[$CO_2$]-T50, E[$CO_2$]-T0, and E[$CO_2$]-T50) with nonparametric two-way ANOVA synchronized permutation tests (B = 1000 permutations) [127]. Nonparametric permutation-based one-way ANOVA followed by pair-wise comparisons with Bonferroni adjustment [127] were performed to compare the means of gene expression values among groups, T0, T25, and T50 (water deficit time - minutes).

## Availability of data and materials

The datasets supporting the conclusions of this article are available in the NCBI BioProject under the accession number PRJNA295411 and within the article and its additional files.

## Authors´ contributions

M.A-F., M.B-M., M.S.B. and A.P.S. planned and supervised the study; M.B-M. and A.P.S. executed the experiments; M.R-A. and M.B-M. contributed to the RNA-seq analysis, categorization and annotations of differentially expressed transcripts; M.B-M. and J.F.S. performed the qPCR assays; M.A-F., M.B-M., A.P.S. and M.S.B. contributed to the interpretation of the data and provided intellectual input; M.B-M. drafted manuscript with contributions of all the authors. All authors read and approved the final manuscript.

## Conflict of interest

The authors declare that they have no conflict of interest.

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## Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.plantsci.2018.12.021.

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